AD ________________

Award Number: DAMD17-01-1-0297

TITLE: Transcriptional Regulation of VEGF Expression in Breast Cancer

PRINCIPAL INVESTIGATOR: Sebastian Bredow, Ph.D.

CONTRACTING ORGANIZATION: Lovelace Biomedical and Environmental Research Institute
Albuquerque, New Mexico 87185

REPORT DATE: June 2002

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)

2. REPORT DATE
June 2002

3. REPORT TYPE AND DATES COVERED
Annual (1 Jun 01 - 31 May 02)

4. TITLE AND SUBTITLE
Transcriptional Regulation of VEGF Expression in Breast Cancer

5. FUNDING NUMBERS
DAMD17-01-1-0297

6. AUTHOR(S)
Sebastian Bredow, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Lovelace Biomedical and Environmental Research Institute
Albuquerque, New Mexico 87185

E-Mail: sbredow@lrri.org

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*
Increased levels of VEGF protein in breast cancer correlate with poor prognosis in the disease. Little is currently known about the transcriptional activation of the gene in breast cancer, and the regulation could be different from other cancer types. To identify promoter segments that contribute to enhanced expression of VEGF in breast cancer, 10 reporter constructs encompassing nested deletions of the wild type-promoter from positions -1200 to -50 were synthesized and screened for activity in human MCF-7 and MDA-MB231 breast cancer cells. Two segments between positions -1200 and -1000 and -270 and -140 were identified as contributors to transcriptional activation of the promoter in these cell lines. Gel shifts using nuclear extracts from MCF-7 and MDA-MB231 cells further indicated differences in transcription factor binding to the proximal promoter element that drives basal expression between positions -88 and -65.

14. SUBJECT TERMS
vascular endothelial growth factor (VEGF), promoter, gene expression transcription, gene therapy

15. NUMBER OF PAGES
10

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

INTRODUCTION

Expression of vascular endothelial growth factor (VEGF) is elevated in malignant cells, and clinical studies correlate increased levels of the protein with poor prognosis in breast cancer (1). Currently little is known about the transcriptional activation of the gene in this particular disease. We hypothesize that regulation of VEGF at the transcriptional level in breast cancer could be different from other types of cancer. Experiments are designed to identify elements in the wild-type (wt) VEGF promoter between positions –1200 and +50 that are responsible for increased transcription in breast cancer cells, and to characterize the transcription factors interacting with these elements. An understanding of how the gene is regulated in this disease could be used as a baseline for developing improved treatment regiments. We plan to synthesize novel expression vectors containing the VEGF promoter elements required for high-level expression in breast cancer cells. Therapeutic genes would thus be expressed specifically in malignant but less so in normal cells.

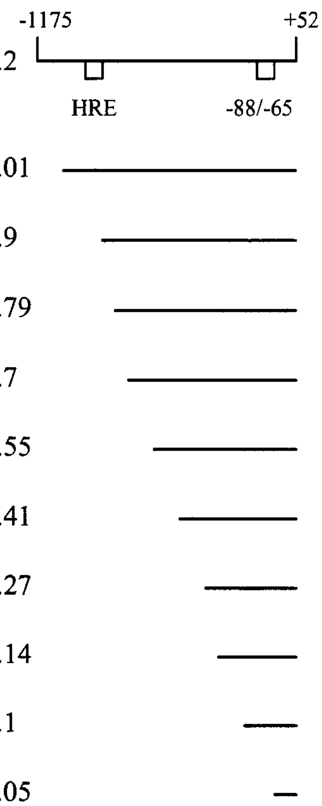
BODY

As outlined in the Statement of Work, year 1 of this proposal was spent identifying *cis*-acting VEGF promoter DNA elements that are responsible for transcriptional activation in transiently transfected breast cancer cell lines. Part of the year was also spent on identifying transcription factors that interact at the functional promoter sites.

Task 1: Identify VEGF promoter elements responsible for the elevated transcriptional activation of the gene in breast cancer cells.

We pursued two approaches to obtain a VEGF promoter fragment with preserved activity. First, attempts to use the polymerase chain reaction (PCR) to amplify the promoter between positions –1175 and +52 were not successful (2, 3). Although several sources for genomic DNA and redesigned primers were used, such modifications did not suffice to overcome the problems usually associated with PCR amplification of GC-rich DNA. Second, in parallel we approached three researchers who previously had described cloning the VEGF promoter, and, in the end, obtained DNA from two of the sources. The plasmid, p2.6, that was used to obtain the deletion constructs described below contains approximately 2.6 kilobases of VEGF promoter, encompassing positions –2362 to +296 (4).

A *SacI/NheI* fragment (-1175 to +52) from p2.6 was subcloned into the luciferase-containing vector pGL2-basic (Promega; Madison, WI), yielding clone p1.2. To obtain progressive promoter deletions at approximately every 100 base pairs in the 5' to 3' direction starting at position -1200, we either used conveniently located restriction sites within p1.2, yielding constructs p1.01, p0.79, p0.27, p0.41, and p0.14 or created the appropriate fragments by PCR (p0.9, p0.7, p0.55, p0.1, and p0.05). We did not obtain constructs containing sequence elements downstream of the proximal GC-rich segment between positions –88 and –65 as most experiments seem to indicate that segments downstream of that position do not contribute to transcriptional activation of the VEGF gene (2, 3, 5, 6). All constructs were verified by sequence analysis.



	MCF-7	MDA-MB231
p1.2	4.52 ± 0.29	12.33 ± 1.75
p1.01	2.52 ± 0.27*	7.26 ± 1.83*
p0.9	2.31 ± 0.12	8.33 ± 1.12
p0.79	2.25 ± 0.31	9.28 ± 0.68
p0.7	3.07 ± 0.51*	13.33 ± 1.38*
p0.55	2.14 ± 0.11*	9.12 ± 2.63*
p0.41	2.57 ± 0.28	13.47 ± 3.34
p0.27	1.62 ± 0.59*¶	4.56 ± 0.58*
p0.14	2.09 ± 0.21	4.02 ± 3.00¶
p0.1	1.12 ± 0.10¶	3.16 ± 1.22
p0.05	0.93 ± 0.35¶	2.19 ± 1.28¶
pGL2-Basic	1.00 ± 0.15	1.00 ± 0.67

Figure 1: Expression of 5' nested VEGF deletions in two breast carcinoma cell lines. Constructs are shown on the left with positions of the hypoxia-response element and proximal promoter indicated by open boxes. Promoter activities were determined by the ratio of firefly luciferase to that of the co-tranfected control RL luciferase and presented as mean ± SD. To determine background, the activity of the promoter-less vector pGL2-Basic was set to one, and the promoter activities of the deletion constructs are displayed as fold pGL2-Basic activity. *$p<0.05$ vs. the next larger construct; ¶$p>0.05$ vs. pGL2-Basic.

The constructs were transfected into two breast carcinoma cell lines (MCF-7, MDA-MB231), which were maintained as per the instructions of the vendor (ATCC; Manassass, VA). As promoter activity was low in MDA-MD231 cells, transfections had to be optimized by testing several transfecting reagents (DOTAP and FuGENE 6, both by Roche Molecular Biochemicals; Indianapolis, IN, and Lipofectamine PLUS by Life Technologies; Grand Island, NY). Internal normalization for transfection efficiency among the cell lines was further achieved by measuring the activity of co-transfected *Renilla reniformis* (RL) luciferase in the same sample using a Dual-Luciferase Reporter® (DLR) kit (Promega). Results of the transfections are summarized in Figures 1.

Several findings emerge from these studies. First, the proximal promoter is not sufficient to drive basal promoter activity; when compared to the promoter-less vector pGL2-Basic, promoter constructs p0.27, p0.14, p0.1, and p0.05 show similar activities. Second, sequences between –1000 and –410 do not seem to contribute to transcriptional activation under normoxic conditions as activities for constructs p1.01, p0.9, p0.79, p0.55, and p0.41 are similar (the approximately 40% increase in promoter activity when sequences between positions –790 and –700 are deleted indicates the presence of a potential negative regulatory element), while deletion of sequences between –410 and –270 reduces promoter strength by 37% in MCF-7 and 66% in MDA-MB231 cells. Third, sequences upstream of position –1000 also contribute to transcriptional activation of the VEGF promoter in breast cancer cells; deletion of these sequences reduces activity by 44% in MCF-7 and 41% in MDA-MB231 cells.

Previous experiments also showed that most cell lines utilize at least a second promoter element in addition to the proximal promoter; the hypoxia-response element (HRE), centered around position –971, activates transcription several-fold in a low oxygen environment (7). We have now optimized the conditions to induce the VEGF promoter under such conditions in breast cancer cells and found that 16 h at 1% O_2 induces a 2.5-fold higher promoter activity of construct p2.6 when compared to control cells that were kept under normoxic conditions (a similar effect can also be observed when cells are incubated with 225 μM $CoCl_2$).

Task 2: Identify and characterize transcription factors that interact at functionally relevant VEGF promoter regions.

To identify the transcription factors that bind to functionally important promoter elements in breast cancer cells, we have begun to investigate the segment constituting the proximal promoter between positions –88 and –65. This fragment, which drives basal promoter activity in many cells, contains binding sites for several transcription factors and binds such factors in a cell line-specific fashion (5, 6, 8, 9). Using a non-radioactive electrophoretic mobility shift assay (LightShift™ Chemiluminescent EMSA Kit; Pierce; Rockford, IL) nuclear extract (3 μg) from MCF-7 and MDA-MB231 cells was incubated with a 5'-biotinylated double-stranded oligonucleotide (Integrated DNA Technologies; Coralville, IA) encompassing positions –88 to –

65. Complexes were resolved on 10% polyacrylamide gels and visualized according to the protocol provided. Results are shown in Figure 2.

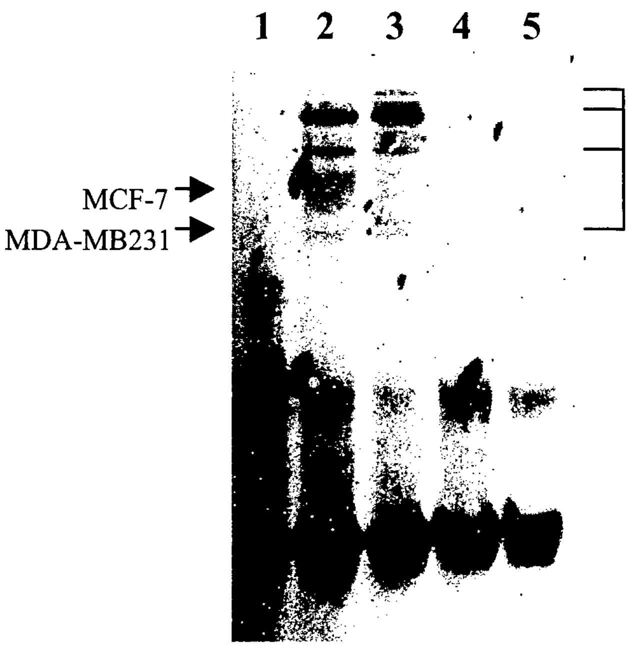


Figure 2: EMSA analyses of transcription factor binding to the proximal VEGF promoter element in MCF-7 (lanes 2 and 4) and MDA-MB231 (lanes 3 and 5) nuclear extracts. The formation of complexes common to both cellular extracts is indicated on the right, while arrows on the left indicate the formation of specific complexes. Competition with 200-fold excess of unlabeled oligonucleotide abolishes binding (lanes 4 and 5). Lane 1 contains free, unbound oligonucleotide DNA.

When incubated with nuclear extracts, constitutive DNA binding activity of several distinct complexes common to both cell lines was observed. In addition, distinct complexes specific for each cell line were also identified. Competition assays using an excess of unlabeled double-stranded oligonucleotide containing the –88 to –65 sequence suppress the formation of the complexes.

KEY RESEARCH ACCOMPLISHMENTS

- Synthesis of 10 VEGF constructs with progressive nested promoter deletions between positions -1200 and –50.
- Analysis of VEGF constructs by transient transfection in two human breast cancer cell lines (MCF-7, MDA-MB231) identified two candidates between positions –1200 and –1000 and –270 and –140, which contribute to transcriptional activation of the promoter.
- Electrophoretic mobility shift assays (EMSA) of nuclear extracts indicate differences in transcription factors binding to the basal promoter element between –88 and –65 in MCF-7 and MDA-MB231 breast carcinoma cells.

REPORTABLE OUTCOMES

Part of this research has been submitted in abstract form and will be presented at the Era of Hope DOD Breast Cancer Research Program Meeting to be held in Orlando, FL, September 25 – 28, 2002.

CONCLUSIONS

The preliminary data indicate known and unknown promoter elements are likely involved in transcriptional activation of VEGF in breast cancer cells. We are currently investigating the promoter activity of the deletion construct in two other human breast cancer cell lines (adenocarcinoma line SK-BR-3 and ductal carcinoma line T-47D) and non-mammary control cells (the human lung carcinoma line A549 and the human mucoepidermoid pulmonary carcinoma line NCI-H292). Experiments are also planned to transfect key constructs into all six cell lines under hypoxic conditions to corroborate the preliminary data. The transfection data from all six cell lines should confirm the presence of regulatory sites unique to breast cancer cells.

The non-radioactive EMSA is currently being optimized. We hope to characterize (and confirm the differences in) factor binding to the proximal promoter element in the upcoming weeks in competition experiments using 1.) different cellular nuclear extracts, 2.) factor site-specific or -mutated oligonucleotides, and 3.) super-shift analyses employing transcription factor-specific antibodies. Experiments are further planned to determine whether a 'scaled up' EMSA yields sufficient complex to allow in-gel enzymatic digests of the protein-DNA complexes for MALDI/TOF-mass spectroscopy peptide mapping.

We have also begun to construct an expression vector that may be used in breast cancer gene therapy. Based on preliminary data, we subcloned the functionally important HRE into a modified form of the expression vector pGWiz (Gene Therapy Systems; San Diego, CA). This modified vector, pGWiz3, contains a minimized human cytomegalovirus promoter/enhancer that otherwise maintains its promoter strength (10). Currently, we are performing transient transfections into breast cancer and control cells to determine the feasibility of such a construct for use in breast cancer gene therapy.

REFERENCES

1. Linderholm, B., Lindh, B., Tavelin, B., Grankvist, K., and Henriksson, R. p53 and vascular-endothelial growth-factor (VEGF) expression predicts outcome in 833 patients with primary breast carcinoma. Int. J. Cancer *89:* 51-62, 2000.
2. Ikeda, E., Achen, M.G., Breier, G., and Risau, W. Hypoxia-induced transcriptional activation and increased mRNA stability of vascular endothelial growth factor in C6 glioma cells. J. Biol Chem., 270: 19761-19766.
3. D'Angelo, G., Ladoux, A., and Frelin, C. Hypoxia-induced transcriptional activation of vascular endothelial growth factor is inhibited by serum. Biochem. Biophys. Res. Commun. *267:* 334-348.

4. Mukhopadhyay, D., Tsiokas, L., and Sukhatme, V.P. Wild-type p53 and v-Src exert opposing influences on human vascular endothelial growth factor gene expression. Cancer Res., *55:* 6161-6165, 1995.
5. Gille, J., Swerlick, R.A., and Caughman, S.W. Transforming growth factor-α-induced transcriptional activation of the vascular permeability factor (VPF/VEGF) gene requires AP-2-dependent DNA binding and transactivation. EMBO J., *16:* 750-759, 1997.
6. Finkenzeller, G., Sparacio, A., Technau, A., Marmé, D., and Siemeister, G. Sp1 recognition sites in the proximal promoter of the human vascular endothelial growth factor gene are essential for platelet-derived growth factor-induced gene expression. Oncogene, *15:* 669-676, 1997.
7. Kimura, H., Weisz, A., Kurashima, Y., Hashimoto, K., Ogura, T., D'Acquisto, F., Addeo, R., Makuuchi, M., and Esumi, H. Hypoxia response element of the human vascular endothelial growth factor gene mediates transcriptional regulation by nitric oxide: control of hypoxia-inducible factor-1 activity by nitric oxide. Blood, *95:* 189-197.
8. Milanini, J., Viñals, F., Pouysségur, and Pagès, G. p42/p44 MAP kinase module plays a key role in the transcriptional regulation of the vascular endothelial growth factor gene in fibroblasts. J. Biol. Chem., *273:* 18165-18172, 1998.
9. Stoner, M., Wang, F., Wormke, M., Nguyen, T., Samudio, I., Vyhlidal, C., Marmé, D., Finkenzeller, G., and Safe, S. Inhibition of vascular endothelial growth factor expression in HEC1A endometric cancer cells through interactions of estrogen receptor α and Sp3 proteins. J. Biol. Chem., *275:* 22769-22779, 2000.
10. Malkoski, S.P., Mobarak, C., and Bredow, S. Augmentation of HCMV promoter expression by a 52-bp hMDR1 promoter element. Molecular Cancer Therapeutics, submitted.

APPENDICES

1.) Copy of Abstract "Transcriptional Response of VEGF in Breast Cancer cells" submitted to the Era of Hope DOD Breast Cancer Research Program Meeting.

Transcriptional Response of VEGF in Breast Cancer Cells

Vascular endothelial growth factor (VEGF) expression is elevated in malignant cells, and clinical studies show a correlation between increased VEGF levels and development and prognosis of breast cancer. Relatively little is known about the transcriptional activation of VEGF in breast cancer, and the regulation of the gene may be distinct not only among different types but also within the same class of tumors. An understanding of how the gene is regulated in this disease could lead to improved treatment regiments: One can potentially design and synthesize novel expression vectors containing only the VEGF promoter elements required for high-level expression in breast cancer. Therapeutic genes would thus be specifically expressed in malignant but less so in normal cells.

Transient transfections of breast cancer cells with a full-length VEGF promoter construct (-1175 to +50) driving the expression of the reporter gene firefly Luciferase have corroborated findings from other cell lines that VEGF expression is inducible by serum starvation irrespective of cell density. We have now introduced 5'-3' deletions every 100 base pairs (bp) into the full-length promoter beginning at position –1010 and ending downstream at position -50 by either utilizing available restriction sites or using polymerase chain reaction (PCR) techniques. These 5' nested deletions will be transiently transfected into a panel of different breast cancer cell lines to identify the promoter elements that cause elevated VEGF breast cancer expression under various stimuli.

The relative shortness of such deletions will allow further characterization of transcriptionally active VEGF promoter fragments in breast cancer cells. Functional gene promoter elements can be cloned directly into promoterless cassettes to determine their activity in malignant and normal cells. Sequences of such elements will be scanned for putative transcription factor binding sites and specific binding to these sites analyzed by electrophoretic mobility shift assay (EMSA) using nuclear extracts. We anticipate that such studies will provide a) a better understanding of the mechanisms underlying the elevated activation of the VEGF gene in breast cancer, and b) a baseline for developing improved gene therapies for this disease.